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Tissue Culture Applications of *Artemisia annua* L. Callus for Indirect Organogenesis and Production Phytochemical

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Key words: Artemisia annua, Indirect organogenesis, Phytochemical, Antioxidant activity

Abstract

Artemisia annua L. axenic plants were used to induce callogeneisis for production of phenolics and plant regeneration. Up to 95% callogenesis from leaf and stem explants on MS supplemented with 2.0 mg/l NAA or 2,4-D + 0.2 mg/l BAP (MSC1 and MSC2) was observed. Lower callus frequency but with improved embryogenic potential was observed upon subculture on medium with reduced auxin and increased BAP concentration (0.5 mg/l NAA or 2,4-D + 0.5 mg/l BAP) (MSC3 and MSC4). The leaf-induced callus on NAA/BAP (MSC3) showed best antioxidant potential. Induced shoot regeneration occurred upon high concentration BAP combined with NAA rather than with 2,4-D (0.25 mg/l NAA or 2,4-D + 1.0 mg/l BAP, MSR 1 and MSR2, respectively). Optimal shoot multiplication and rooting were obtained on 1.0 mg/l BAP and 0.1 mg/l IBA, respectively, followed by acclimatization of regenerants to greenhouse conditions. This work aims at establishing protocol for *A. annua* preservation and biosynthesis of natural products.

Introduction

Artemisia annua L. (Asteraceae) has been used for over two millennia in traditional Chinese medicine for a variety of complaints - from skin disorders to high blood pressure and malaria (Aftab et al. 2018). Qinghaosu or artemisinin (a sesquiterpene) was discovered as an antimalarial drug and treatments containing an artemisinin derivative have now been standardized for the treatment of malaria worldwide. Researchers have gathered comprehensive information about the medicinal properties of *A. annua* that have

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allowed it to be used against various diseases because of its antimalarial, immunosuppressive, anti-inflammatory, anticancer, antihypertensive, antioxidative, antimicrobial, antiparasitic, insecticidal, and antiviral properties. It has been recognized that the therapeutic effects are due to the synergistic contribution of multi-components, not just by the major constituents (Han et al. 2008). In support, antioxidant phenolic phyto-constituents of *A. annua* were suggested to play role in its anti-inflammatory activity in human intestinial cells (de Magalhães et al. 2012), in resistance to *Plasmodium falciparum* malaria parasites (Suberu et al. 2013), against cancer metastasis (Ko et al. 2016).

Plant biotechnological *in vitro* techniques have been considered as powerful tools for conservation of germplasm and multiplication of *Artemisia* sp. for research and therapeutic tasks (Ali et al. 2007). Recently, we developed an efficient protocol for mass micropropagation of *A. annua* where the antioxidant potential of extracts from *in vivo*, *in vitro* and *ex vitro* adapted plants was compared (Zayova et al. 2018). The data highlighted that during growth in *in vitro* conditions the antioxidant parameters became significantly reduced, while subsequent re-growth back in soil restored the biological capacity.

In the last decade, there is a focus on development of plant cell factories representing a potential renewable source of valuable natural products (Yordanova and Georgiev 2017, Marchev et al. 2020). Several studies demonstrated the capacity of *Artemisia* sp. callus cultures to produce phenolic compunds: in *A. absinthium* (Ali and Abbasi 2014), *A. scoparia* (Yousaf et al. 2019). The main purpose of this study was to establish protocol for efficient callogenesis from stem and leaf explants of *A. annua* axenic plants, and to monitor the ability of the obtained callus variants to produce phenolic compounds (total phenolics and flavonoids) and the respective antioxidant activity. On the basis of callus cultivation, an optimal regeneration procedure was developed for indirect shoot organogenesis, multiplication of regenerants and their *ex vitro* adaptation in soil.

Materials and Methods

For callus induction, explants (stem and leaves) were detached from one-month-old *Artemisia annua* L. axenic plants (Zayova et al. 2018). Each explant was cut into three pieces and placed on to 150/20 mm cultivation tubes containing 10 ml MS (with 3.0% sucrose) supplemented with PGRs such as NAA or 2,4 D + BAP (MSC1 and MSC2) (Table 1). Callus induction occured under controlled conditions (22 ± 2°C, 70% humidity, 16/8 hrs photoperiod, 40 μmol/m^{2}s illumination by Philips 36 W cool white fluorescent tubes) and scored after four weeks cultivation. The calluses were further subcultivated on fresh MS containing NAA or 2,4 D + BAP (MSC3 and MSC4) (Table 1). After three subcultures, the callus characteristics were scored.

For indirect organogenesis, the leaf-derived calluses on MSC3 and MSC4 media were transferred on PRG-free medium as a control (MSR0) and on media supplemented with NAA or 2,4 D + BAP (MSR1 and MSR2) (Table 1). The shoot regeneration was evaluated after four weeks. For shoot multiplication, the regenerated plants were cultivated on MS

with three different BAP concentrations for four weeks (Table 1). For rooting, *A. annua* regenerants were transferred to half strength MS (½MS) (with 2.0% sucrose (control variant) and to IBA containing medium for rooting for three weeks (Table 1).

Table 1. Composition of nutrient medium variants.

Nutrient medium	Variants	PGRs (mg/l)			
		NAA	2,4-D	BAP	IBA
Callus	MS0	0	0	0	
induction	MSC1	2.0	-	0.2	
	MSC2	-	2.0	0.2	
	MSC3 (subculture)	0.5	-	0.5	
	MSC4 (subculture)	-	0.5	0.5	
Shoot	MSR0	0	0	0	
regeneration	MSR1	0.25	-	1.0	
	MSR2	-	0.25	1.0	
Shoot				0.5	
multiplication				1.0	
				1.5	
Rooting					0
on ½ MS					0.1
medium					0.5

For *ex vitro* acclimatization, *A. annua* regenerants were moved to a growth chamber (24 ± 1°C, 16/8 hrs photoperiod, 50 μmol/m^2 s illumination by fluorescent lamps). They were planted individually to small plastic pots (8 cm diameter) containing mixture of soil : perlite : sand (2 : 1 : 1, v/v/v) covered with a transparent polythene membrane to ensure high humidity (90%) and then opened after three weeks. The survival rate of the acclimatized plants was detrmined after six weeks. After two months, the plants were transferred to a greenhouse.

The total phenolic content in the callus tissues was determined by the Folin-Ciocalteu method (Singleton et al. 1999) and gallic acid was used the reference standard compound. The flavonoid content was measured after the aluminium chloride colorimetric method (Chang et al. 2002) with quercetin used to create the standard curve. The total antioxidant activity (TAA) was measured according to Prieto et al. (1999) using α-tocopherol as a reference.

Each treatment involved at least 20 samples and the data are presented as mean ± SE. The morphometric data were statistically analyzed using ANOVA for comparison of means, and significant differences were calculated according to LSD test at 5% significance level (Statgraphics Plus sowftware, version 5.1 for Windows). The antioxidant parameters were analysed by one Way ANOVA followed by Holm-Sidak

statistical test (Sigma Plot 11.0 software) at 5% significance level. The Pearson correlation coefficient (R) was calculated using the average parameter values for the variants (n = 8) at 5% significance level.

Results and Discussion

The callus cultures are the preferred type of plant *in vitro* cultures, and they are used also in *Artemisia* sp. to produce healthy biomass under controlled growth conditions from which medicinally potent natural products could be extracted (Ali et al. 2017, Yousaf et al. 2019). Several tissue culture protocols for callus culture, organogenesis and cell culture of *A. annua* have been well established (Alejos-Gonzalez et al. 2013, Mohammad et al. 2014, Dangash et al. 2015, Ghassemi et al. 2015, Tahir et al. 2015, Mirzaee et al. 2016). Previous reports on *A. annua* showed that the type of explants used as callus source significantly affect callus- and organogenesis (Alejos-Gonzalez et al. 2013, Ghassemi et al. 2015). In the present study, the effects of NAA and 2,4-D and BAP on callogenesis from stem and leaf explants of *A. annua* axenic plants were investigated (Table 2, Fig. 1). All the combinations NAA/BAP and 2,4-D/BAP promoted callus formation. The highest frequency of callus formation (95% for leaf and 85% for stem explants) was observed when high auxin and low BAP concentration was applied - 2.0 mg/l NAA or 2,4-D + 0.2 mg/l BAP (MSC1 and MSC2). In this case, the calluses were green with friable structure (Fig. 1 a, b, e, f). Lower frequency of callogenesis but with improved embryogenic potential was observed upon subculture on medium with reduced auxin and increased BAP concentration - 0.5 mg/l NAA or 2,4-D + 0.5 mg/l BAP (MSC3 and MSC4). The calluses were compact green and yellowish with granular hard appearance (Fig. 1 c, g). The frequency of callus induction on all the four media was high but leaf explants were more responsive than the stem ones. A tendency for increased callus fresh biomass was detected for both explants in the presence of 2,4-D (MSC2 and MSC4), while the dry biomass was comparable between the variants and it was strongly reduced in stem-derived callus on 2,4-D.

Both, leaf- and stem-derived callus tissues contained higher quantity of phenolics upon the combination NAA/BAP (MSC1 and MSC3), and significantly less when 2,4-D was used (MSC2 and MSC4) (Table 2). The content of total flavonoids in all experimental variants remained extremely low and it seemed independent from the type of auxin and more abundant in stem explants. The TAA correlated better with the total phenolics ($R = 0.764$, $p < 0.027$) and no correlation with the total flavonoids was found. The highest content of phenolics and TAA was measured in calluses grown on MSC3 and MSC1 media.

In many circumstances when compared with the natural plants, callus cultures established in the presence of different PGRs have been found to produce important metabolites in bulk (Benjamin et al. 2019). Plant biotechnology offers a sustainable method for the bioproduction of plant secondary metabolites using *in vitro* systems

(Marchev et al. 2020). The effective extraction and obtaining higher yields of herb-derived metabolites are challenged by factors such as plant stage of development, plant culture, extraction procedure, etc. (Mohammad et al. 2014). In authors previous report, they explored quantitive antioxidant indicators among *A. annua* plants grown in their natural habitat, *in vitro* cultivated plants (regenerated directly from shoot tip explant) and *ex vitro* acclimitazed plants (adapted from *in vitro* conditions back to soil). It was observed that

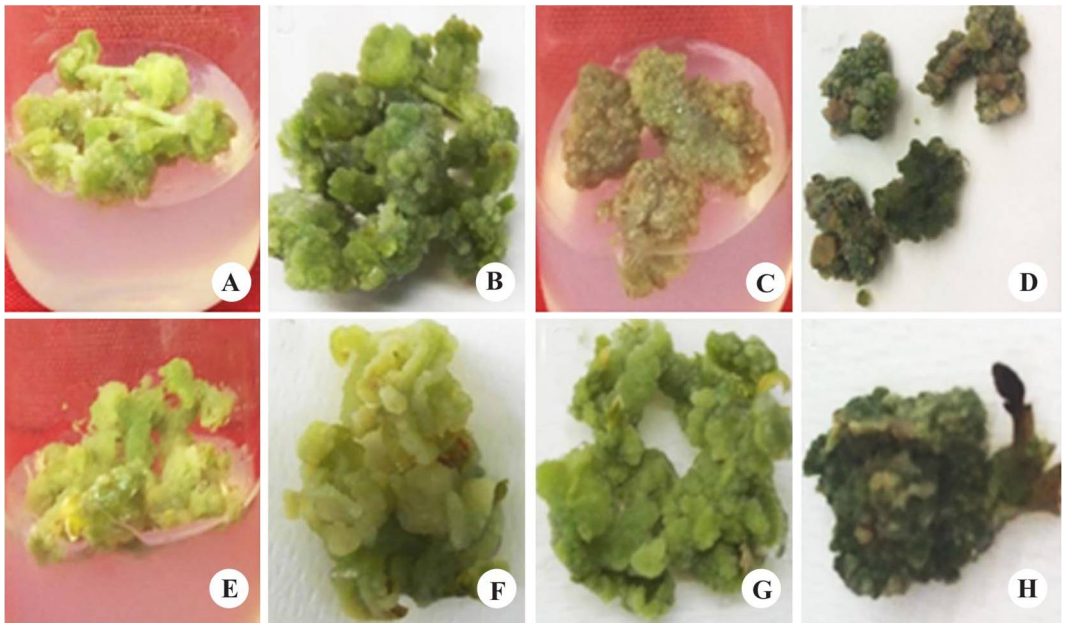

Fig. 1a-h. Callus induction and indirect shoot organogenesis of *A. annua:* (a) and (b) Green, friable callus from stem explants on MSC1, (c) Green granular callus from stem expants on MSC3, (d) Dark green granular callus with indirect shoot organogenesis from stem explants on MSR1, (e) and (f) Green, friable callus from leaf explants on MSC1, (g) Green granular callus from leaf explants on MSC3, (h) Dark green granular callus with indirect shoot organogenesis from leaf explant on MSR1.

during *in vitro* cultivation the phenolics content and TAA were reduced with nearly 50%. During the *in vitro* cultivation of regenerated plants, the antioxidant parameters increased by 10 - 30% when the medium was supplemented with 0.1 mg/l IBA and the effect increased by reducing the sucrose from 3.0 to 2.0%. The antioxidant parameters varied as following - 40 - 50 mg/g DW for phenols, 7 - 8 mg/g DW for flavonoids and 65 - 80 mM/g DW for TAA. In the present study, the callus variants produced between 28 - 72 mg/g DW phenols, 1.5 - 3 mg/g DW flavonoids and 4 - 21 mM/g DW TAA. We concluded that in comparison to the *in vitro* grown plants, the callus cultures provided higher quantity of total phenols, however the flavonoid level and TAA decreased. It would be very informative to monitor additional biological activities to estimate the capacity of this tissue culture type for use as a plant cell factory. Reports showed that in callus cultures the type and concentrations of PGRs modulate antioxidant capacity and additional

supplements as biotic (yeast, fungi) and abiotic (calcium chloride, light) elicitors could enhance the accumulation of phenolics: up to 8.5 mg/g DW phenolics in *A. absinthium* (Ali and Abbasi 2014); up to 12 mg/g DW phenolics and 10 mg/g DW flavonoids in *A. absinthium* (Tariq et al. 2014); up to 5.4 mg/g DW phenolics and 4.2 mg/g

Table 2. Effect of PGRs on callus induction from stem and leaf explants of *A. annua* axenic plants, and respective antioxidant parameters of the callus.

Medium variants	Frequency of callus (%)	Callus characteristic	FW (g)	DW (g)	Phenols, (mg/g DW)	Flavonoids, (mg/g DW)	TAA, (mM/g DW)
Stem							
MSC1	80	Green Friable	1.124	0.157	71.0 ± 1.6^{a}	2.9 ± 0.1^{a}	19.4 ± 1.3^{a}
MSC2	85	Green Friable	1.299	0.095	30.0 ± 2.1^{b}	2.8 ± 0.2^{a}	4.4 ± 0.2^{c}
MSC3	75	Green Granular	1.217	0.136	69.0 ± 2.1^{a}	2.4 ± 0.3^{ab}	11.8 ± 0.6^{b}
MSC4	70	Yellowish Granular	1.129	0.085	28.2 ± 2.0^{b}	1.7 ± 0.1^{b}	10.5 ± 1.2^{b}
Leaf							
MSC1	95	Green Friable	1.079	0.152	65.0 ± 2.2^{a}	1.6 ± 0.1^{a}	14.3 ± 1.0^{b}
MSC2	90	Green Friable	1.931	0.145	52.0 ± 3.6^{b}	1.6 ± 0.1^{a}	17.2 ± 1.1^{a}
MSC3	85	Green Granular	1.005	0.148	72.0 ± 2.1^{a}	2.0 ± 0.2^{a}	20.6 ± 1.7^{a}
MSC4	80	Yellowish Granular	1.780	0.162	52.0 ± 1.8^{b}	1.5 ± 0.2^{a}	12.7 ± 1.2^{b}

The data are presented as means of an average sample of 20 plants per treatment ± SE. Different letters indicate significant differences assessed by Holm-Sidak test (5%) after performing one way ANOVA multifactor analysis.

DW flavonoids in *A. monosperma* (Al-Gendy et al. 2016). These published data in comparison with present results on *A. annua* highlight the efficiency of the species and the established callus cultures for production of phenolics. As has been reported, the flavonoid content could be elvated by elicitors such as yeast and high concentrations (1 - 4 mg/l) of TDZ (Tariq et al. 2014). Mohammad et al. (2014) observed analogical tendency for much lower artemisin content (~ 0.006% DW) in developed friable, green calli derived from leaves of *A. annua* compared to the maximum artemisinin accumulation (~1.0%

DW) in most of the field grown strains of *A. annua* at or near the onset of flowering, thereby, restricting the plant to be harvested only once annually. Elicitor application such as *Aspergillus* sp. increased artemisinin content of *A. annua* calli from 0.0036 - 0.028% DW (Yuliani et al. 2018).

Table 3. Effect of plant growth regulators on *A. annua* regeneration from callus derived from leaf explants.

Medium variants	Parameters		
Shoot regeneration*	Shoot regeneration (%)	Number of shoots per leaf-derived callus	
MSR1	30	1.3 ± 0.11[a]	
MSR2	15	0.9 ± 0.07[b]	
Shoot multiplication*	Shoot formation (%)	Number of shoots per regenerant	Shoot length (cm)
0.5 mg/l BAP	60	2.0 ± 0.17[a]	1.2 ± 0.10[b]
1.0 mg/l BAP	85	4.3 ± 0.31[bc]	1.5 ± 0.13[b]
1.5 mg/l BAP	75	2.6 ± 0.22[c]	1.3 ± 0.12[b]
Rooting	Root formation (%)	Number of roots per regenerant	Root length (cm)
0	45	0.8 ± 0.07[c]	1.0 ± 0.16[a]
0.1 mg/l IBA	90	1.7 ± 0.14[ab]	2.6 ± 0.21[b]
0.5 mg/l IBA	60	1.5 ± 0.12[b]	1.6 ± 0.15[a]

Data is presented as means of 20 plants per treatment ± SE. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing ANOVA multifactor analysis. *The control parameters are 0.

Since the leaf-derived callus was more responsive to subsequent shoot induction than the stem one, we focused on shoot regeneration from leaf-derived callus (Table 3). The induced calluses were transferred to media for stimulation of shoot regeneration by increased BAP concentration – 0.25 mg/l NAA or 0.25 mg/l 2,4-D + 1.0 mg/l BAP (MSR1 and MSR2), which was favourable for both types of explants (Fig. 1d, h). Callus proliferation significantly increased after subculturing of the regenerated callus. The frequency of indirect shoot organogenesis and the number of shoots per callus occurred on MSR1 (30% and 1.3 shoots), followed by MSR2 (15% and 0.9 shoots), which suggested NAA as more appropriate to use for shoot organogenesis than 2,4-D (Table 3). The 2,4-D is a synthetic hormone that in addition to its auxin-like activity is believed to affect the plant developmental processes by inducing the stress responses (Wójcik et al. 2020), while the effect of NAA seems not to be so strong. Other studies also support that NAA is preferred for shoot organogenesis than 2,4-D (Tahir et al. 2015, Mirzaee et al. 2016). On the other hand, the effect of cytokinins on *A. annua* shoot induction from callus, specially

BAP and TDZ, was recognized in previous studies, as well (Alejos-Gonzalez et al. 2013, Mohammad et al. 2014, Dangash et al. 2015, Ghassemi et al. 2015, Tahir et al. 2015, Mirzaee et al. 2016).

To multiply the number of shoots, the regenerated *A. annua* shoots were further cultivated on MS containing cytokinin at different concentrations (0.5, 1.0 and 1.5 mg/l BAP) for the stage of micropropagation (Table 3, Fig. 2a-c). The maximum frequency of shoot formation (85%) was observed on 1.0 mg/l BAP, which induced the maximum number of shoots (4.3) with longest shoot (1.5 cm) after four weeks of cultivation (Fig. 2c). For root induction, the regenerated shoots were rooted after transferring to ½MS supplemented with 2.0% surcose (control) and with 0.1 mg/l and 0.5 mg/l IBA (Table 3). The control variants showed 45% root formation, while the best parameters were detected in the medium containing 0.1 mg/l IBA - up to 90% rooting and increased number of roots (1.7), with average root length of 2.6 cm per regenerated plant (Fig. 2d). The results showed that ½ MS with 0.5 mg/l IBA reduced root formation (60%). In support, Ganesan and Paulsamy (2011) suggest IBA as most suitable PGR in inducing roots compared to IAA and NAA.

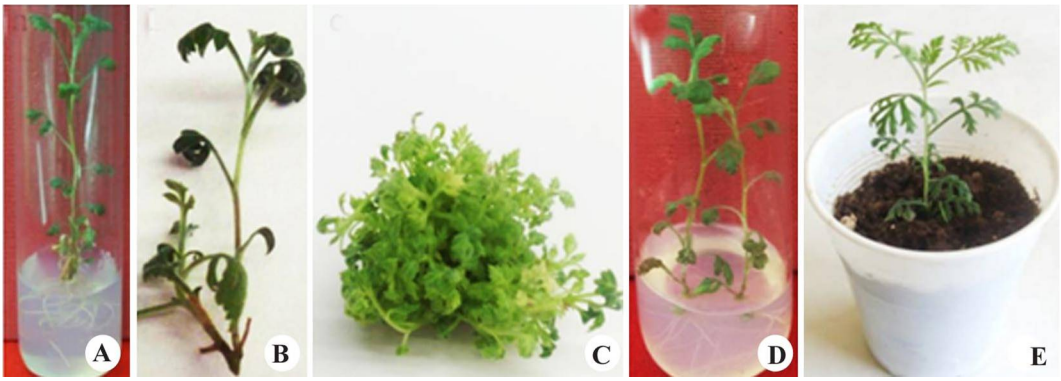

Fig. 2a-e). Micropropagation of *A. annua* regenerated plants: (a) and (b) regenerated plant, (c) *in vitro* regenerated propagated plant on MS with 1.0 mg/l BAP, (d) *in vitro* regenerated rooted plants on ½ MS with 0.1 mg/l IBA, (e) *Ex vitro* acclimatized plant.

The regenerated *in vitro* plants revealed a good adaptability *ex vitro* on potting mixture consisted of soil, perlite and sand (2 : 1 : 1 v/v/v), which was found to be the most appropriate for *A. annua ex vitro* adaptation (Fig. 2e). The survival of *A. annua* plants under *ex vitro* conditions was evaluated as 45% of regenerants. During the adaptation, the plants continued to grow and develop, which is considered as an indication of successful *ex vitro* acclimatization. The plants were then transferred to greenhouse conditions. The lower rate of *ex vitro* acclimation compared to previous studies (approx. 45% vs. 65 to 90%) could be explained with the explant origin from *in vitro* plants and not from *in vivo* plants, which suggests effect of endogenous hormone level (Ganesan and Paulsamy 2011, Hailu et al. 2013,Zayova et al. 2018). Regenerated *in vitro* and *ex vitro*

adapted plants could also serve as a source for plant extracts enriched in useful metabolites (Alejos-Gonzalez et al. 2013, Zayova et al. 2018).

The established, in the present study, protocol highlighted that *A. annua* leaf explants in combination with NAA/BAP PGRs could generate embryogenic callus with higher quantity of phenolics compared to *in vitro* plants but with reduced level of flavonoids and antioxidant activity. This work could serve as a base for further detailed analysis of the modulation of the phytochemical potential of tissue cultures. It concluded that the *in vitro* plant technology is of great importance to preserve *A. annua* plants from extinction and to upscale the production of natural products on industrial level.

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